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POTENTIAL USE OF MANGIFERA INDICA SEED KERNEL AND CITRUS AURANTIIFOLIA SEED IN WATER DISINFECTION

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ABSTRACT

High cost of chemical disinfectants used in water treatment is one of the most crucial factor rendering water treatment expensive, not only which, the health risk posed by these chemicals are also of concern to many researchers. Hence, exploration of natural products as alternative disinfectant is a growing research area. In this study, sodium chloride extract of mangnifera indica (Duncan mango) seed kernel and citrus aurantiifolia (key lime) seed were investigated using agar well diffusion and micro broth dilution methods for their antibacterial activity against some enteric pathogenic organisms; Staphylococcus aureus (ATCC25923), Enterococcus faecalis (ATCC29212); Escherichia coli (ATCC25922), Klebsiella spp. (clinical strain), Pseudomonas spp. (ATCC4853), and Salmonella typhi (clinical strain). Results revealed that the extracts have antimicrobial activity against the test organisms. In agar well diffusion method, the extracts were most effective at concentration 100mg/ml. In the broth dilution method, the extracts were bacteriostatic at lower concentration and bactericidal at higher concentration. Key lime seed extract was found to have the least minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on Escherichia coli compared with chlorine, hence it was more potent on Escherichia coli than chlorine in liquid broth medium. The antimicrobial activity of the extracts against the test organisms were comparable to that of chlorine. The study revealed that Duncan mango seed kernel and key lime seed extracts have antimicrobial properties which can be made more effective as that of chlorine, thus this will be relevant in water disinfection and will improved water treatment in poor developing countries. It is concluded that the extracts have potential use in water disinfection.

Keywords: *Disinfection, Mangifera indica seed kernel, Citrus aurantiifolia seed, Chlorine, Seed extract*

1. INTRODUCTION

Most parts of the world especially developing countries uses surface water for drinking purposes. Water from such source is easily contaminated by pathogenic organisms (mainly sheltered by suspended particles contaminants) due to its exposed nature. Pathogenic organisms' contaminants in drinking water are known to transmit diseases such as diarrhoea, cholera, dysentery, and typhoid and it is estimated that such contaminants in drinking water cause about 502,000 worldwide diarrhoeal deaths annually [1]. Hence, water treatment is required to remove or destroy pathogenic organisms before using the water for drinking purposes. A particular method of water treatment is chosen depending on the quality of raw water obtained and the use to which the water is to be put. Disinfection is the most widely used method to remove pathogenic organisms from water, and this is achieved through the addition of disinfectants. In conventional water treatment process, oxidizing chemicals such as chlorine is most widely used because of its effectiveness, durability and availability. However, the use of chlorine has some drawbacks associated with it. Most developing countries spend huge amount of money to import chlorine, chlorine has the potentials of forming carcinogenic and mutagenic

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disinfection by-products [2], the sludge produce from water treatment with chlorine is not eco-friendly [3], chlorine decays as a result of natural evaporation in some areas of the distribution system leading to loss of chlorine concentration along the water distribution system [4]. Chemical disinfectants are of public health concern, as they are associated with increased risk of cardiovascular diseases, cancers and birth defects [5, 6, 7]. All these drawbacks associated with chemical disinfectants makes it crucial to look for alternatives to these chemicals for water treatment.

Natural products especially plant extracts can be considered as alternatives to chemical disinfectants for water treatment, hence they are presumed safe, readily available and cheap [8]. *Moringa oleifera* seeds extract has been found to inhibit the growth of *E.coli* [3, 9].Also *Plantago ovate* [10] and Mustard seeds [11] have been found to possess antibacterial activity. Mango (*Mangifera indica*) belongs to the family of Anacardiaceae. It is found widely in Bangladesh, India and Pakistan where it is indigenous and cultivated varieties have been introduced to other warm regions in the world including Sub-Sahara African. The ripe fruit is fleshy, edible and it covers a single seed. Mango seed kernels are rich in carbohydrates, proteins, fat, minerals and vitamins, hence can be used as potential source of nutrients for human and animal feed [12]. Mango seed kernel also contains considerable amount of phenolic compound [13] as well as high levels of antioxidant [14]. Antibacterial screening of ethanol, methanol, acetone and phosphate extract of some varieties of mango seed kernel (blackgold, lemak and waterlily) showed antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa* [13]. Key lime (*Citrus aurantiifolia)* is a small globose fruit belonging to Rutaceae family, it is cultivated in tropical and warm subtropical areas as a commercial crop and is seedier than the regular lime. Citrus fruits can serve as source of nutrients for human as it contains appreciable amount of ascorbic acid, phenolic compounds and pectin [15 – 18]. Citrus fruit juice and seeds contain phytonutrients such as polyphenols, anthocyanins and hydroxycinnamic acids which are useful to human health and they have been reported to possess antioxidant and antimicrobial properties [19].

Considering the draw backs associated with the use of chemical disinfectants for water treatment, sourcing for alternative disinfectants is essential and is currently a growing research area. As mentioned earlier, naturally occurring disinfectants can be considered as alternative to chemical disinfectants and plant could serve as a better lead into the development of these disinfectants as they contain secondary metabolites that protect them from pathogens. To achieve this aim, there should be a combined effort devoted through continuous research to screen more plants for antimicrobial properties which could be developed into effective water disinfectant. This study was aimed at evaluating the potential use of *Mangifera indica* (Duncan mango) seed kernel and *Citrus aurantiifolia* (key lime) seed in water disinfection. The specific objectives of the study were:

(i) To screen for antimicrobial activity of Duncan mango seed kernel and key lime seed extracts.
(ii) To analyse the minimum inhibitory concentration (MIC) of the extracts on pathogenic organisms.
(iii) To analyse the minimum bactericidal concentration (MBC) of the extracts on pathogenic organisms.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Fresh ripe fruits of Duncan mango and key lime were purchased from a local market (Uselu) in Benin City, Edo State, Nigeria. The fruits were identified and authenticated by a Botanist in the Department of Plant Biology and Biotechnology, University of Benin, Benin City.

2.2 Processing of the Plant Materials

The fruits were washed thoroughly with distilled water and air-dried, after which they were sliced open using a stainless steel knife. Duncan mango seed kernels and key lime seeds were removed manually and air-dried. They were then pulverised mechanically and sieved manually into fine powder.

2.3 Extraction of Plant Materials

The seed and seed kernel powders were extracted with ethanol (96%) using Soxhlet extraction method and the cakes were placed in the oven at 105^{o}C for about 6 to 7 hours to evaporate the solvent. These were further extracted individually using sodium chloride to obtained final concentrations of 50mg/ml and 100mg/ml. These concentrations were prepared by adding 0.5g and 1g separately of each cake into 0.1M sodium chloride solution (0.58g of sodium chloride powder was added to 10ml of distilled water and stirred properly using a magnetic stirrer for 10 minutes to completely dissolve the salt powder), the suspensions were mixed using a magnetic stirrer for 10 minutes and then filtered using What Man Number 1 equivalent filter paper. The filtrates were used as the

seed extracts. Similar procedure was carried out by dissolving 1/2 chlorine tablet (0.5g of chlorine powder) and 1 chlorine tablet (1g of chlorine powder) separately into 10ml of distilled water to also obtain 50mg/ml and 100mg/ml concentrations. Fresh seed extracts and chlorine solution were prepared at every day of use.

2.4 Preparation of the Culture Media

The culture media were prepared according to manufacturer's specification as follows:

2.4.1 Nutrient Broth Media

Single and double strength nutrient broth media were prepared. For preparation of the single strength nutrient broth media; about 13g of nutrient broth powder was dissolved in 1 Litre of distilled water inside a Schott bottle (1L), it was properly mixed and distributed into test tubes. These test tubes were plugged with a cotton wool and then sterilized by autoclaving at 121^{o}C for 15minutes. Similar procedure was used for preparation of the double strength nutrient broth media by dissolving 26g of the nutrient broth powder into 1Litre of distilled water.

2.4.2 Nutrient Agar Media

About 28g of nutrient agar powder was suspended in 1Litre of distilled water inside a Schott bottle which was plugged with cotton wool and brought to boil in order to dissolve the powder completely. It was sterilized by autoclaving at 121^{o}C for 15minutes and then cool in a water bath at 45^{o}C for 15minutes.

2.5 Preparation of Test Micro-organisms (Stock Solution)

The test organisms used in this study were obtained from Microbiology Laboratory, Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi and they are; *Staphylococcus aureus* (ATCC25923), *Enterococcus faecalis* (ATCC29212), *Escherichia coli* (ATCC25922), *Klebsiella spp.* (clinical strain), *Pseudomonas spp.* (ATCC4853), and *Salmonella typhi* (clinical strain). Stock solution of individual test organism was prepared. Test organisms were cultured in a single strength nutrient broth media to obtain stock solutions; 0.1ml (100µl) of individual test organism was inoculated into 10ml of the liquid broth inside separate test tubes, the test tubes were plugged with cotton wool and incubated at 37^{o}C for 24 hours. These microbial suspensions were further prepared to 0.5 McFarland standard by double fold serial dilution to obtain standardized microbial suspensions.

2.6 Antimicrobial Activity

The extracts were screened for Antimicrobial activity using Kirby-Bauer agar well diffusion method, while the micro broth dilution method was used to analyse minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts.

Antimicrobial Susceptibility testing*:* The antibacterial activity for each seed extract against individual test organism; *Staphylococcus aureus* (*S.aureus*), *Enterococcus faecalis* (*E. faecalis*),*Escherichia coli*(*E. coli*), *Klebsiella spp.* (*Klebsiella*), *Pseudomonas spp.* (*Pseudomonas*) and *Salmonella typhi*(*S. typhi*) was carried out using Kirby-Baueragar well diffusion method. About 0.5ml (5µl) of the McFarland standard microbial suspensions were inoculated into 20mlof the molten nutrient agar in separate test tubes, they were mixed properly and poured into sterile plates (90mm diameter) and then allowed to solidify at room temperature. Wells were bored in the agar plates using a sterile cork borer (no. 7). The wells were filled with 0.2ml of 50mg/ml or 100mg/ml concentrations of the seed extracts and chlorine solution. The plates were left for 30minutes for effective diffusion of the extracts and chlorine solution into the agar and then incubated at 37^{o}C for 24 hours. The zone of inhibition was determined by taking the average of the diameters of the zone of growth inhibition (a clear region around the plate with the antimicrobial agent on the agar surface) if any measured with a transparent ruler. The standard mean error was calculated form the standard deviation of the diameter zone of inhibition determined. The experiments were done in replicates to ensure consistency.

2.6.1 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of the extracts were determined by the micro broth dilution method using 96 well micro-titre plates [20]. A double fold serial dilution of the extracts were made using distilled water to obtain the following concentrations; 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml,1.5625mg/ml, 0.78125mg/ml. Each well in the micro-titre plates was inoculated with equal volume of each type of extract and double strength nutrient broth (100µl) or chlorine solution and double strength nutrient broth (100µl) , followed by the standardized micro-organisms (10µl) making a total volume of 210µl on each well. A micro-titre plate with wells containing double strength nutrient broth and standardized micro-organisms, double strength

nutrient broth without organisms serves as positive and negative controls. The micro-titre plates were immediately incubated at 37°C for 24 hours. The lowest concentration of each type of extract and chlorine solution that inhibited microbial growth was observed and recorded as the MIC. This was indicated by the absence of purple colouration after 30 minutes upon the addition of 3-(4, 5-dimethylthiazol -2-yl) -2, 5-diphenyltetrazoliumbromide (MTT) solution to the micro-titre wells after the 24 hours incubation period. The experiments were also done in replicates to ensure consistency.

2.6.2 Minimum Bactericidal Concentration (MBC)

New micro-titre plates were inoculated with fresh double strength nutrient broth and samples from each micro-titre plates that showed no visible growth from the MIC test. Micro-titre wells containing only double strength nutrient broth serve as negative control to check the sterility of the media. These micro- titre plates were immediately incubated at 37°C for 24 hours. The minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent that results in the death of the micro organisms. Therefore, the lowest concentrations of each type of extracts and chlorine solution yielding no growth was considered as the MBC. Experiments were done in replicates to ensure consistency.

3. RESULTS AND DISCUSSIONS

Results of the antimicrobial susceptibility testing, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are presented. Below Table1 shows result of antimicrobial activity of the seed extracts against test organisms. The seed extracts exhibited antimicrobial activity against most of the test organisms. Result also revealed that as concentration of the seed extracts increases, the inhibitory effect also increases, thus indicating more active components in the seed extracts. This is in agreement with results reported from previous findings on antibacterial effect of plant extracts [21], hence the seed extracts were more effective at concentration of 100mg/ml.At this concentration, values of inhibitory zone diameter of the seed extracts ranges from 17 to 22.7 mm which is a considerable range compared to that of chlorine (20.5 to 26.5 mm). *Escherichia coli, Salmonella typhi*, *Pseudomonas spp.* and *Enterococcus faecalis* were found to be susceptible to both Duncan mango and key lime seed extracts. *Klebsiella Spp.* and *Staphylococcus aureus* showed resistance against key lime seed extract. On a solid medium bacteria grow on the surface and concentration gradient could develop during incubation leading to pseudo resistance of bacteria [22]. *Staphylococcus aureus* was more susceptible to Duncan mango seed extract as large inhibition zone diameter was obtained.

These seed extracts clearly inhibited the growth of *Escherichia coli*. This is an indication that the seed extracts can be used as desirable tools in the control of undesirable microorganisms in water treatment. Studies have revealed that certain bioactive components in plants are known to exert antimicrobial activity [23]. Different varieties of mango seed kernel extracts (black gold, waterlily, lemak) and citrus plants have been reported to have antibacterial activity against pathogenic organisms(*Staphylococcus aureus, Bacillus subtilis, Escherichia coli and pseudomonas aeruginosa)* [13, 24].

Table 1: Antimicrobial Activity of Seed Extract against Test Organisms

Seed Extracts/ Concentration (mg/ml)	Test Organisms/ Mean Diameter Zone of Inhibition (mm)±SEM Against					
	E.coli	*S.typhi*	*Klebsiella*	*Pseudomonas*	*E.faecalis*	*S.aureus*
DMS						
100	18.50±0.29	17.00±0.58	19.5±0.29	20.00±0.0	20.65±0.78	22.7±0.17
50	na	na	na	19.35±0.38	na	na
KLS						
100	17±0.58	18±0.58	na	19±0.58	19.6±0.81	na
50	13±0.58	na	na	na	na	na
Chlorine						
100	26.5±0.29	23.5±0.87	25±0.58	22.5±0.29	20.5±0.29	25±0.58
50	23.5±0.29	22±0.0	22±0.0	20.5±0.29	21±0.0	22±0.0

na = No activity; SEM = Standard error mean; DMS = Duncan mango seed; KLS = Key lime seed

Table 2: Minimum Inhibitory Concentration Assay

Seed Extracts	Test Organisms	Concentrations (mg/ml)							
		100	50	25	12.5	6.25	3.125	1.5625	0.78125
DMS	*E.coli*	-	-	+	+	+	+	+	+
	S.typhi	-	-	+	+	+	+	+	+
	Klebsiella	-	-	-	-	+	+	+	+
	Pseudomonas	-	-	-	-	-	-	+	+
	E.faecalis	-	-	-	+	+	+	+	+
	S.aureus	-	-	-	+	+	+	+	+
KLS	*E.coli*	-	-	-	-	-	+	+	+
	S.typhi	-	-	-	+	+	+	+	+
	Klebsiella	-	-	+	+	+	+	+	+
	Pseudomonas	-	-	-	-	+	+	+	+
	E.faecalis	-	-	-	+	+	+	+	+
	S.aureus	-	-	+	+	+	+	+	+
Chlorine	*E.coli*	-	-	-	-	+	+	+	+
	S.typhi	-	-	-	-	+	+	+	+
	Klebsiella	-	-	-	+	+	+	+	+
	Pseudomonas	-	-	-	-	-	-	+	+
	E.faecalis	-	-	-	-	+	+	+	+
	S.aureus	-	-	-	-	+	+	+	+
Positive Control	*E.coli*	+	+	+	+	+	+	+	+
	S.typhi	+	+	+	+	+	+	+	+
	Klebsiella	+	+	+	+	+	+	+	+
	Pseudomonas	+	+	+	+	+	+	+	+
	E.faecalis	+	+	+	+	+	+	+	+
	S.aureus	+	+	+	+	+	+	+	+
Negative Control	*E.coli*	-	-	-	-	-	-	-	-
	S.typhi	-	-	-	-	-	-	-	-
	Klebsiella	-	-	-	-	-	-	-	-
	Pseudomonas	-	-	-	-	-	-	-	-
	E.faecalis	-	-	-	-	-	-	-	-
	S.aureus	-	-	-	-	-	-	-	-

+= Indicate growth; -= Indicate no growth

Table 3: Minimum Inhibitory Concentration (MIC) of Seed Extracts

Seed Extracts	Test Organisms/ MIC (mg/ml)					
	E.coli	*S.typhi*	*Klebsiella*	*pseudomonas*	*E.faecalis*	*S.aureus*
DMS	50	50	12.5	3.125	25	25
KLS	6.25	25	50	12.5	25	50
Chlorine	12.5	12.5	25	3.125	12.5	12.5

Table 2 and Table 3 showed result of minimum inhibitory concentration assay and minimum inhibitory concentration (MIC) of seed extracts. Results indicated that the activities of the seed extracts were concentration dependent. Positive control showed that the medium without the seed extracts/chlorine had no inhibitory effect on bacterial growth and negative control showed that the media was not contaminated with bacteria. From Table 3, result revealed that the seed extracts were bacteriostatic against all test organisms. In liquid broth medium, there are more cell to antimicrobial compound contact as the bacteria is submerged in the plant extracts-containing medium [22]. Values ranges from 3.125 to 50 mg/ml, indicating considerable MIC range compared to those of chlorine (3.125 to 25mg/ml). The disparity between the activities of the seed extracts and chlorine may be due to the mixture of bioactive compounds present in the seed extracts compared to the pure compound contained in the standard disinfectant (chlorine) [25, 22]. Some of the MIC values of the seed extracts were lower than or same as that of chlorine. The MIC of key lime seed extract (6.25 mg/ml) require to inhibit the growth of *Escherichia coli* was much lower than that of chlorine (12.5 mg/ml) and the MIC of Duncan mango seed extract (12.5 mg/ml) required to inhibit the

growth of *Klebsiella* was also much lower than that of chlorine (25 mg/ml). Also, the MIC of Duncan mango seed extract (3.125 mg/ml) required to inhibit the growth of *Pseudomonas* was the same as that of chlorine (3.125 mg/ml). Thus, these results suggest that the seed extracts contained active antimicrobial agent that could be comparable to that of chlorine and as such can be useful in water disinfection. Table 4 and Table 5 showed results of minimum bactericidal concentration (MBC) assay and minimum bactericidal concentration (MBC) of the seed extracts.

Negative control further showed that the media was not contaminated with bacteria. Result from Table 5 indicated that the seed extracts were bactericidal against all test organisms with concentrations ranging from 12.5 to 100 mg/ml. Again it was revealed that the MBC value of key lime seed extract (25 mg/ml) on *Escherichia coli* was much lower than that of chlorine (50 mg/ml) and the MBC of Duncan mango seed extract (50 mg/ml) on *Klebsiella* and *Staphylococcus aureus* was the same as that of chlorine (50 mg/ml).

Results (Table 3 and 5) clearly showed that MIC values were lower than MBC values, suggesting that the seed extracts were bacteriostatic at lower concentration and bactericidal at higher concentration. These results (Duncan mango seed extract) are in agreement with those reported from previous findings on antibacterial activity of mango kernel extracts [13]. It was deduced from the results (Table 3 and 5), that the seed extracts possess highly efficient antibacterial activity against enteric pathogenic organisms and can be used as an excellent natural antibacterial agent in drinking water treatment.

Table 4: Minimum Bactericidal Concentration Assay

Seed Extracts	Test Organisms	Concentrations (mg/ml)							
		100	50	25	12.5	6.25	3.125	1.5625	0.78125
DMS	*E.coli*	-	+	nd	nd	nd	Nd	nd	nd
	S.typhi	-	+	nd	nd	nd	Nd	nd	nd
	Klebsiella	-	-	+	nd	nd	Nd	nd	nd
	Pseudomonas	-	-	-	-	+	+	nd	nd
	E.faecalis	-	-	+	nd	nd	Nd	nd	nd
	S.aureus	-	-	nd	nd	nd	Nd	nd	nd
KLS	*E.coli*	-	-	-	+	+	Nd	nd	nd
	S.typhi	-	-	nd	nd	nd	Nd	nd	nd
	Klebsiella	-	+	nd	nd	nd	Nd	nd	nd
	Pseudomonas	-	-	-	+	nd	Nd	nd	nd
	E.faecalis	-	-	+	nd	nd	Nd	nd	nd
	S.aureus	-	+	nd	nd	nd	Nd	nd	nd
Chlorine	*E.coli*	-	-	+	+	nd	Nd	nd	nd
	S.typhi	-	-	-	+	nd	Nd	nd	nd
	Klebsiella	-	-	+	+	nd	Nd	nd	nd
	Pseudomonas	-	-	-	-	-	+	nd	nd
	E.faecalis	-	-	-	+	nd	Nd	nd	nd
	S.aureus	-	-	+	+	nd	Nd	nd	nd
Negative Control	*E.coli*	-	-	-	-	-	-	-	-
	S.typhi	-	-	-	-	-	-	-	-
	Klebsiella	-	-	-	-	-	-	-	-
	Pseudomonas	-	-	-	-	-	-	-	-
	E.faecalis	-	-	-	-	-	-	-	-
	S.aureus	-	-	-	-	-	-	-	-

+= Indicate growth; -= Indicate no growth; nd= not determined

Table 5: Minimum Bactericidal Concentration (MBC) of the Seed Extracts

Seed Extracts	Test Organisms/MBC(mg/ml)					
	E.coli	*S.typhi*	*Klebsiella*	*pseudomonas*	*E.faecalis*	*S.aureus*
DMS	100	100	50	12.5	50	50
KLS	25	50	100	25	50	100
Chlorine	50	25	50	6.25	25	50

5. CONCLUSION

This study demonstrated that Duncan mango seed kernel and key lime seed extracts possess antimicrobial activity against some enteric pathogens (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella* and *Pseudomonas*). The extracts showed antimicrobial activity comparable to that of chlorine. Thus, these extracts could be made more effective as chlorine and will be relevant in water disinfection. This will help improve water treatment in poor developing countries particularly rural communities where there are difficulties assessing inorganic disinfectants. The plant species that produces Duncan mango and key lime seeds are widely grown, so the seeds will be readily available and cheap. These seeds are presumed to be safe since they are of natural origin. It is therefore concluded that Duncan mango seed kernel and key lime extracts have potential use in water disinfection.

6. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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